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Report

The E3 Ubiquitin Ligase BIG BROTHER Controls *Arabidopsis* Organ Size in a Dosage-Dependent Manner

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Summary

Organ growth up to a species-specific size is tightly regulated in plants and animals [1, 2]. Final organ size is remarkably constant within a given species, suggesting that a species-specific size checkpoint terminates organ growth in a coordinated and timely manner. Phytohormones influence plant organ size [3–7], but their precise functions in size control are unclear because of their pleiotropic and complex developmental roles [8–10]. The *Arabidopsis* transcription factors AINTEGUMENTA and JAGGED promote organ growth by maintaining cellular proliferation potential [11–16]. Loss of the *Antirrhinum* transcription factor CINCINNATA causes leaf overgrowth, yet also leads to a highly abnormal leaf shape [17]. Thus, no dedicated factor that limits the final size of plant organs has been isolated. Here, we identify the novel RING-finger protein BIG BROTHER (BB) as a repressor of plant organ growth. Small changes in *BB* expression levels substantially alter organ size, indicating a central regulatory role for BB in growth control. Recombinant BB protein has E3 ubiquitin-ligase activity that is essential for its in vivo function, suggesting that BB acts by marking cellular proteins for degradation. Our data indicate that plants limit the duration of organ growth and ultimately organ size by actively degrading critical growth stimulators.

Results and Discussion

BIG BROTHER Encodes a RING-Finger Protein

To identify repressors of organ growth, we have characterized two mutant alleles of a novel *Arabidopsis* gene, termed *BIG BROTHER* (*BB*), that cause the formation of larger-than-normal floral organs (Figures 1A, 1C, and 1D). The *bb-1* mutation involves a chromosomal rearrangement at the lower end of chromosome III, including a deletion of the *At3g63530* locus and the two neighboring genes (Figure S1A in the Supplemental Data available online). Transformation of *bb-1* mutants with a wild-type transgene containing only the *At3g63530* locus completely rescued the mutant phenotype (Figures 1C–1E). The *bb-2* T-DNA insertion allele in *At3g63530* has strongly reduced *At3g63530* mRNA levels and enlarged floral organs (Figures 1C, 1D, and 1H). Thus, *At3g63530* is the *BB* gene.

BB encodes a protein of 248 amino acids that contains a RING-finger domain of the H2 type at its C terminus [18] (Figure 1B). Many RING-finger-containing proteins act as E3 ubiquitin ligases at the specificity-determining step of the ubiquitination cascade, with the RING-finger binding to E2 ubiquitin-conjugating enzymes (see below) [19]. Proteins that share significant homology with BB outside the RING finger are found only in plants (Figure S2), suggesting that BB fulfills a plant-specific function in size control.

BB Limits Organ Size in a Dosage-Dependent Manner

We examined a series of genotypes that express increasing amounts of *BB* mRNA from the endogenous promoter, ranging from 0% to 600% of the wild-type level (Figures 1A and 1C–1H). Homozygous *bb-1* mutants, which lack *BB* mRNA (Figure 1H), form larger petals and sepals, as well as thicker stems than wild-type (Figures 1C–1E), and occasionally an enlarged gynoecium with more than two carpels (data not shown). Also, they accumulate more biomass in the form of flowers than wild-type plants (Figure 1E). Heterozygous *bb-1/BB* plants, which contain approximately half the wild-type amount of *BB* mRNA (Figure 1H), have an intermediate phenotype (Figures 1C–1E). *bb-2* mutants, which express approximately 20% of the wild-type *BB* mRNA level (Figure 1H), closely resemble *bb-1/BB* plants (Figures 1C–1E).

Transgenic *bb-1* mutants expressing wild-type levels of *BB* mRNA from a genomic rescue construct (RL1) are very similar to wild-type plants (Figures 1C–1F and 1H). By contrast, moderate or strong *BB* overexpression from this construct in lines RL2 and RL4 (~300% or 600% of wild-type levels, respectively) leads to a correspondingly moderate or strong reduction in petal and sepal size, stem thickness, and flower biomass accumulation (Figures 1C–1E and 1H). Plants overexpressing *BB* from the viral *35S* promoter show very similar phenotypes (Figures S3A–S3D).

Leaves are strongly reduced in size in RL2 and RL4 plants that overexpress *BB*, but are not enlarged in genotypes with reduced *BB* function (Figure 1F). *bb-1* leaves are in fact significantly wider than wild-type leaves, yet also slightly shorter, giving an unaltered leaf area. Thus, leaf size in *bb-1* mutants may be limited by redundant genes or physiological factors, for example nutrients, consistent with previous observations [20].

Cell size is at most weakly influenced by *BB* expression levels, except in RL4 plants, which have fewer

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and smaller cells (Figures 1C and 1F), indicating that *BB*-dependent changes in organ size and biomass are mainly due to altered numbers of normally sized cells. Because cell growth is generally a prerequisite for cell division [21, 22], *BB* may primarily repress cell growth and thus indirectly limit cell numbers. This idea is supported by the reduced cell size in RL4 plants, where excess *BB* activity appears to inhibit cell growth to the point where the normal coordination between growth and division is perturbed.

In summary, *BB* is both necessary and sufficient to limit *Arabidopsis* floral organ size, floral biomass accumulation, and stem thickness, acting in a strictly dosage-dependent manner over a range of physiological concentrations (0%–600% of wild-type levels). In particular, the *bb-2* phenotype and the haploinsufficiency of the *bb-1* mutation suggest that *BB* is a limiting component of the organ-size checkpoint in flowers and the stem, underscoring the need to precisely regulate *BB* expression levels to attain normal organ sizes.

BB Restricts the Duration of Proliferative Growth

To determine how *BB* limits organ size, we performed a kinematic analysis of petals in *bb-1* mutants and in *BB*-overexpressing plants relative to wild-type. Plotting the sizes of petal primordia over time reveals that the organ enlargement in *bb-1* mutants is not due to a higher rate of growth than in wild-type plants (Figure 2A; estimated exponential-growth coefficients of 0.0177 for both wild-type and *bb-1*). Rather, *bb-1* mutant petals continue growing for a longer period of time (Figure 2A). This prolonged growth phase is accompanied by a 20% increase in the time interval separating successive flowers in *bb-1* plants (Figure 2A; note the larger time interval between successive data points for the mutant), suggesting that *BB* function is also required for normal plastochron length.

To determine how cell division and cell expansion contribute to the observed growth dynamics of petal primordia, we measured the mitotic index and cell size over time in wild-type and *bb-1* mutant petals [23]. Cells in *bb-1* petals do not divide faster, but rather continue to proliferate for a longer time and begin to enlarge later than wild-type petal cells (Figure 2B).

Conversely, petal primordia from the *BB*-overexpressing line RL4 grow for a shorter period of time at an essentially wild-type rate (Figure 2C; exponential-growth coefficients of 0.0216 for wild-type and 0.0205 for RL4). Thus, *BB* limits organ size by restricting the duration of the proliferative growth phase, rather than the rate of growth.

BB Acts Independently of *ANT* and *JAG*

A prolonged period of organ growth also underlies the organ enlargement in *AINTEGUMENTA* (*ANT*)-overexpressing plants [13], suggesting that *ANT* and *BB* could function antagonistically in a common pathway. To test this, we analyzed the mRNA expression levels of *BB* in *ant* mutants and *35S::ANT*-overexpressing plants and of *ANT* and its downstream target *CycD3;1* [13] in plants with varying *BB* activity. *BB* mRNA levels do not show robust changes in ant^{72F5} mutants and *35S::ANT* plants (Figure S4). Similarly, the levels of both *ANT* and *CycD3;1* mRNA are at most very weakly affected by alterations in *BB* activity (Figure 1G), as are the mRNA levels of two additional cell-cycle regulators (*CyclinB1;1* and *ICK1/KRP1* [24, 25]; data not shown) and of *JAGGED* (*JAG*) (Figure 1G).

To test for genetic interactions between *BB* and *ANT* or *JAG*, we measured petal sizes in the respective double mutants. The phenotype of ant^{72F5} *bb-1* double mutants was essentially additive, suggesting that the two genes act in independent pathways (Figure 2D).

Like *jag-1* single-mutant petals, *bb-1 jag-1* double-mutant petals lacked the distal lamina (Figure S5A). However, the width of the remaining proximal petal stalk was equally enlarged by the *bb-1* mutation in *JAG* wild-type and *jag-1* mutant backgrounds (Figure S5B). Thus, the *jag-1* mutation is epistatic to *bb-1* with respect to ablation of the distal petal lamina, yet *BB* still operates in the remaining petal stalk. Although this formally makes *JAG* a candidate for repression by BB, we favor the alternative hypothesis that the two genes act sequentially, with *JAG* first allowing formation of the distal petal domain, whose growth is then modulated by *BB* activity. Supporting this, overexpression of *JAG* in developing flowers from the *APETALA1* promoter did not increase organ size [12, 14], making it unlikely that elevated *JAG* activity underlies the organ overgrowth of *bb* mutants. Thus, *BB* appears to act in a novel pathway, independent of *ANT* and *JAG*.

BB Is Expressed in All Proliferating Tissues

By RT-PCR, *BB* mRNA can be detected in all plant organs tested (Figure S7B). RNA in situ hybridization on seedlings and inflorescences reveals *BB* expression mainly in proliferating tissues, i.e., shoot and floral meristems and young organs, and in the vasculature (Figures 3A–3C). During petal growth, *BB* expression is gradually restricted to the distal part of the petal (Figures 3D–3F), where cells continue proliferating longest [12], and eventually disappears (Figure 3G). This expression pattern in the proliferating regions of the shoot is confirmed by the GUS staining pattern in *pBB::GUS* plants (Figures 3H and 3I). The *BB* promoter is also active in the root apical meristem and vasculature (Figure 3J) and in developing embryos (Figures 3K and 3L). Thus, *BB* is expressed in all actively growing regions of the plant and in the vasculature.

The BB Protein Is Rapidly Turned over by Proteasomal Degradation, and Its Abundance Correlates with Cell Proliferation

To study the distribution of BB protein, we expressed a BB-GUS translational fusion under the control of the *BB* promoter in homozygous *bb-1* mutants. The fusion protein fully rescues the *bb-1* mutant phenotype (Figure 4G), demonstrating its functionality. The *pBB::BB-GUS* construct produces the same expression pattern as the promoter-only *pBB::GUS* construct, yet the staining intensity is strongly reduced (Figures 4A–4C). Inhibiting the proteasome in *pBB::BB-GUS*-expressing plants by epoxomicin increases the accumulation of the BB-GUS fusion protein (Figure 4D), without upregulating *BB-GUS* mRNA expression (Figure S6A). This increase is suppressed by cotreatment with the translational inhibitor cycloheximide, which abolishes GUS staining (Figure 4E), indicating that it reflects accumulation of

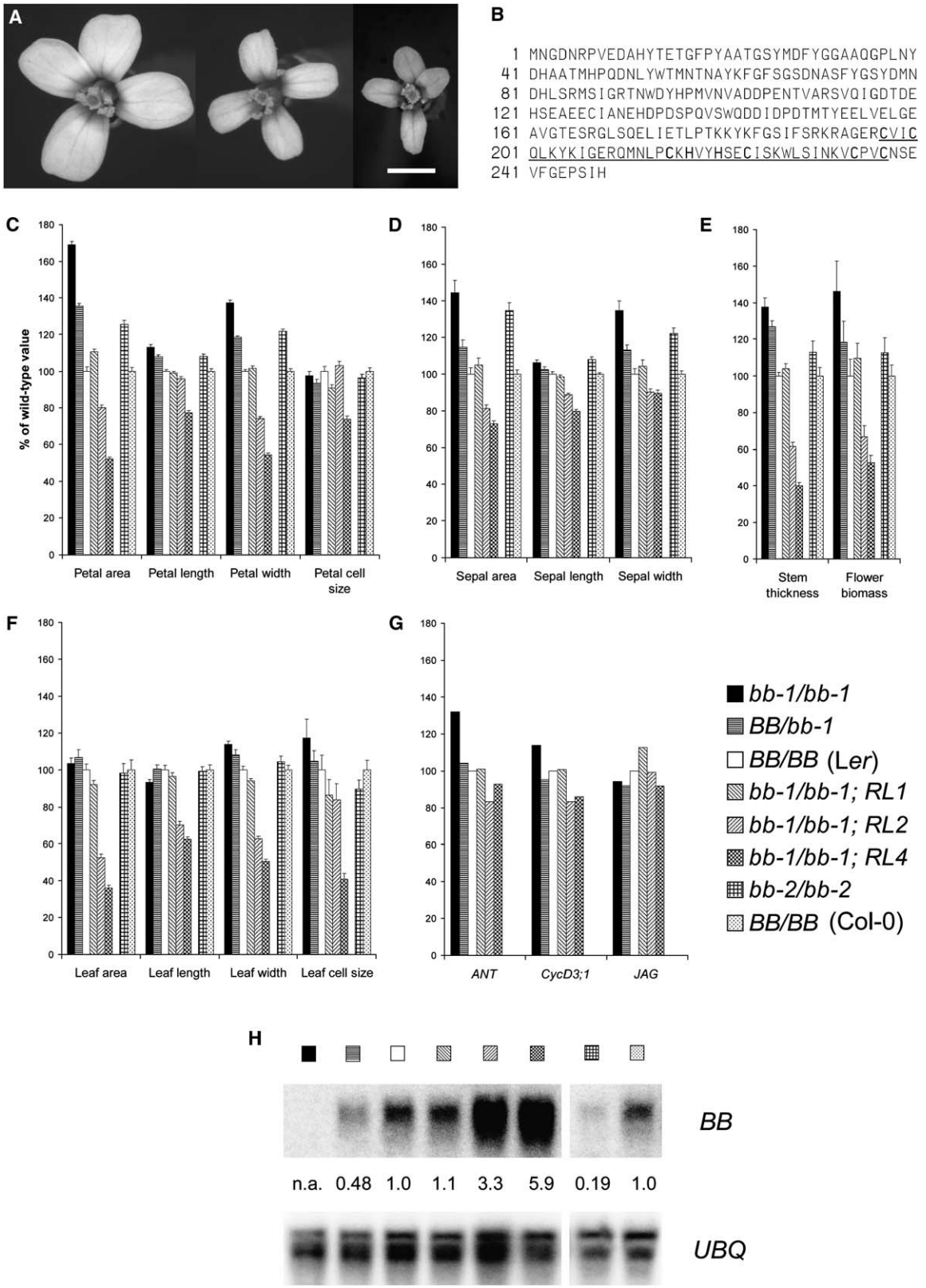


Figure 1. Dosage-Dependent Control of Organ Size by *BB*

(A) Homozygous *bb-1* mutant (left), L*er* wild-type (middle), and *BB*-overexpressing flower from RL4 (right). Scale bars: 1 mm.

(B) BB amino acid sequence. The RING finger is underlined, with the eight conserved cysteines and histidines in bold.

(C–F) Floral organ (C and D) and leaf (F) sizes, stem thickness, and floral biomass accumulation (E) as a function of *BB* activity. Values are given as mean + standard error of the mean (SEM) relative to the respective wild-type value set at 100%. Flower biomass is fresh weight of flowers formed

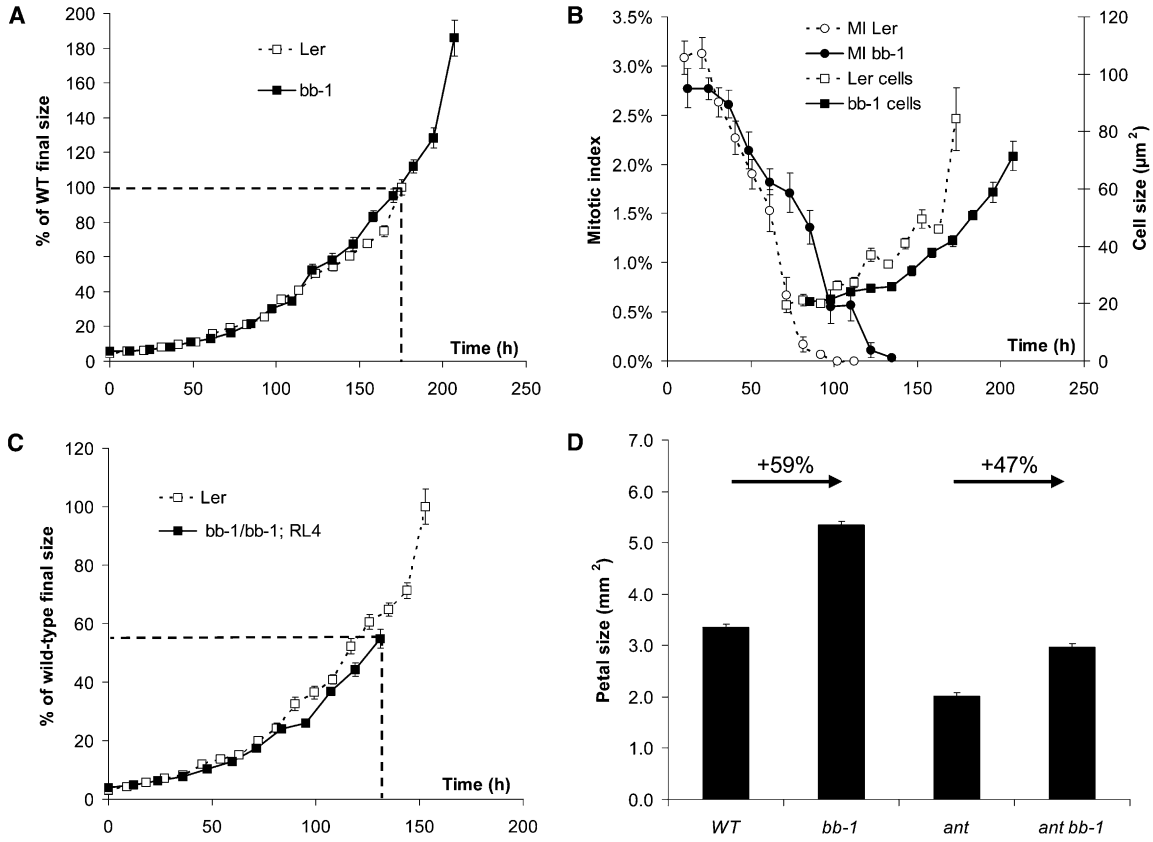


Figure 2. Kinematic Analysis of Petal Growth

(A) Growth of wild-type and *bb-1* mutant petal primordia over time. Dashed lines indicate the end points of the wild-type curve. The largest petals of each series are from the oldest unopened flower buds. See Supplemental Experimental Procedures for details.

(B) Mitotic index (MI) and cell size over time in wild-type and *bb-1* mutant petals. Time axis in (B) corresponds to the one in (A).

(C) Growth of wild-type and *BB*-overexpressing petal primordia over time. Dashed lines indicate the end points of the curve for the overexpressing RL4 line.

(D) Petal size in ant^{72F5} *bb-1* double and respective single mutants. Percentages indicate the relative size increase due to loss of *BB* function. In a second independent experiment, these values were +64% in *bb-1* versus wild-type and +53% in *bb-1* ant^{72F5} versus ant^{72F5}. Error bars indicate SEM.

newly synthesized fusion protein. The staining intensity in plants either carrying a *pBB::GUS* transgene or expressing an inactive form of BB protein fused to GUS ($BB^{C197S,C200S}$, see below) does not change when treated with epoxomicin (Figure S6B), indicating that the observed effect is specific for the fusion protein of wild-type BB to GUS. Thus, the BB-GUS protein is produced in actively growing and proliferating regions of the plant and is rapidly turned over by proteasome-mediated degradation.

To analyze the temporal relationship between BB protein expression and cell division activity in more detail, we compared the GUS staining patterns of *bb-1* mutant petals expressing *pBB::BB-GUS* to those of wild-type petals expressing the mitotic marker *pCycB1;1::CDBGUS* over time [23]. BB-GUS protein is present from early developmental stages onward in the proliferating regions of petal primordia, and BB-GUS levels and cell division activity decline with very similar kinetics during petal growth (Figures 4H and 4I). These data confirm that BB protein is expressed throughout the phase of cell proliferation and therefore suggest that *BB* acts by gradually diminishing the cells' capacity for further growth and cell division.

Given the effects of phytohormones on organ growth, we tested whether any of the major classes of phytohormones (auxin, cytokinin, gibberellin, brassinosteroids, ethylene, abscisic acid, jasmonic acid) could influence transcription from the *BB* promoter or accumulation of BB protein. The GUS staining of neither *pBB::GUS*- nor *pBB::BB-GUS*-expressing plants was affected by treatment with any of these substances (data not shown).

per plant per day. The ratio of fresh weight to dry weight was very similar for *bb-1* mutants, wild-type, and *BB*-overexpressing plants (12.6%, 13.3%, and 11.8%, respectively). Results of statistical tests are shown in Table S1. Legend to the right of panel (G) refers to the entire figure.

(G) mRNA expression levels of selected growth markers as determined by northern blotting on total RNA from inflorescences with mixed stage flowers. Signals as detected by a phosphorimager were normalized to the polyubiquitin hybridization signals on the same filters, and the values indicated are relative to wild-type set at 100%.

(H) A northern blot of total RNA from inflorescences with mixed-stage flowers was sequentially probed for *BB* and for polyubiquitin genes as loading control. The values indicate the relative intensity of the *BB* hybridization signal as detected by a phosphorimager normalized to the ubiquitin signal. Symbols above the lanes correspond to (C–G) and the legend. RL denotes rescue line; n.a. denotes not applicable.

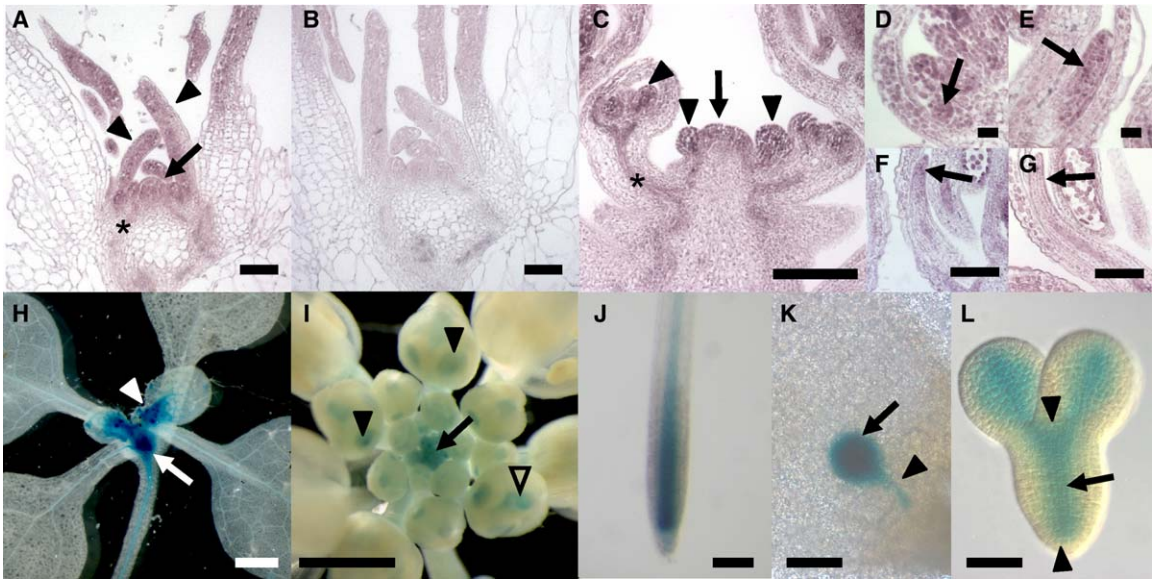


Figure 3. Expression Pattern of *BB*

(A–G) mRNA in situ hybridization with *BB* antisense probe to wild-type (A and C–G) or *bb-1* mutant (B) tissue.
(A) Seedling; shoot meristem (arrow), developing vasculature (asterisk), and young leaf primordia (arrowheads) are indicated.
(B) *bb-1* mutant seedling.
(C) Inflorescence; shoot meristem (arrow), young floral meristems and inner organs of developing flowers (arrowheads), and vasculature (asterisk) are indicated.
(D–G) *BB* expression during petal development (stages according to [26]). From stage 7 (D) to 9 (E) *BB* is expressed uniformly. *BB* expression becomes restricted to the distal part of the petal by stage 10 (F) and disappears after stage 11 (G). Arrows indicate petal primordia.
(H–L) *BB* promoter activity as monitored by the *pBB::GUS* transgene.
(H) GUS staining in the seedling shoot apex and young leaf primordia (arrow). GUS staining follows cell division activity in developing leaves [27] (arrowhead).
(I) GUS staining in the inflorescence shoot apex and young flower primordia (arrow), in developing petals (closed arrowheads) and gynoecia (open arrowhead) of older flowers.
(J) GUS staining in the root meristem and vasculature.
(K and L) GUS staining in the globular embryo (arrow) and suspensor (arrowhead) (K), and later in the developing vasculature (arrow) and apical meristem regions (arrowheads) (L).
Scale bars represent the following: 100 μm in (A)–(C) and (J), 10 μm in (D) and (E), 50 μm in (F), (G), (K), and (L), and 500 μm in (H) and (I).

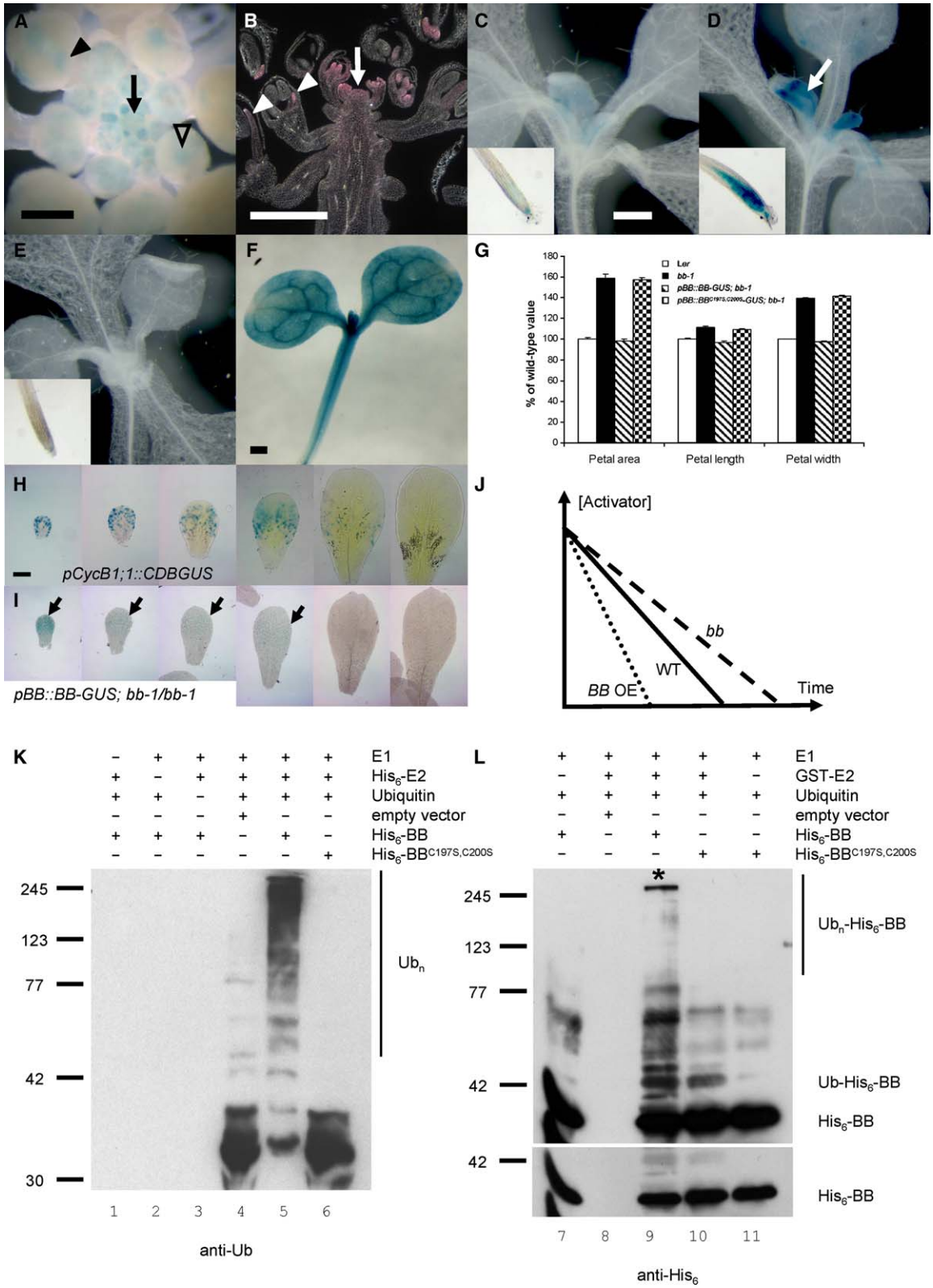
Also, *bb-1* mutants did not show detectably altered sensitivity to the tested phytohormones compared to wild-type plants (data not shown). Thus, *BB* appears to act independently of the major phytohormones.

BB Is a Functional E3 Ubiquitin Ligase

The presence of a RING-finger domain in the BB protein suggests that it acts as an E3 ubiquitin ligase to mark cellular proteins for proteasomal degradation. Consistent with this, we find that integrity of the RING finger is required for the biological activity of BB-GUS protein and for its rapid proteasomal degradation: A construct expressing a fusion protein between GUS and a mutant form of BB with two conserved RING-finger cysteines changed to serines (C197S,C200S) from the endogenous *BB* promoter produced very strong GUS staining

Figure 4. Characterization of BB Protein

(A–F) GUS staining of homozygous *bb-1* mutant plants carrying a *pBB::BB-GUS* (A–E) or *pBB::BB*^{C197S,C200S}*-GUS* (F) transgene.
(A and B) Whole-mount (A) and longitudinal section (B) through inflorescences show GUS staining in the shoot meristem and young floral meristems (arrow), in gynoecia (open arrowhead) and petals (filled arrowheads) of developing flowers. Samples were stained four times longer than in Figure 3I. Reaction product appears purple in dark-field (B).
(C–E) Inhibition of the proteasome by epoxomicin (D) increases GUS staining (arrow) compared to controls (C). This increase is blocked by co-treatment with cycloheximide (E). Insets show seedling roots.
(F) C197S,C200S RING-finger mutations increase GUS staining in the absence of proteasomal inhibition.
(G) The BB^{C197S,C200S}-GUS protein does not rescue the petal overgrowth of *bb-1* mutants. Values are indicated as mean + SEM relative to the L*er* wild-type value set at 100%. Open bars show L*er* wild-type; filled bars show *bb-1* mutants; hatched bars show *pBB::BB-GUS; bb-1*; and checkered bars show *pBB::BB*^{C197S,C200S}*-GUS; bb-1*.
(H and I) GUS-stained petal primordia from *pCycB1;1::CDBGUS* (H) or *bb-1; pBB::BB-GUS*-expressing plants (I). Arrows indicate staining in *bb-1; pBB::BB-GUS* petals. The youngest petals (left) are from stage-8 to stage-9 flowers, the oldest (right) from stage-11 flowers.
(J) Model for *BB* function in organ size control. Organs grow until the level of growth activators falls below a critical threshold as a result of BB-mediated degradation. In *bb* mutants, the activator level decreases more slowly, allowing prolonged growth and formation of larger organs. Conversely, *BB* overexpression (*BB* OE) reduces the activator level more quickly, leading to earlier growth termination and smaller organs.
(K and L) In vitro ubiquitin-ligase assays. Blots were detected with anti-ubiquitin (K) or anti-His$_6$ (L) antibodies.



(K) His_6-tagged BB protein catalyzes the formation of high-molecular-weight ubiquitin chains (Ub_n). The intense band above 30 kDa in lanes 4 and 6 most likely represents short ubiquitin chains formed independently of E3 activity in the assay. "empty vector" indicates protein from a mock purification from a culture containing only the empty His_6-expression vector.

(L) His_6-tagged BB protein undergoes autoubiquitination. The lower panel in (L) shows a shorter exposure. The asterisk denotes modified His_6-BB fusion protein formed in the presence of E1, E2, and ubiquitin that was unable to enter the separating gel.

Size markers on the left are in kDa.

Scale bars represent 300 μm in (A)–(C) (the bar in [C] applies also to [D] and [E]) and (F), and 100 μm in (H) (where the bar also applies to [I]).

in the absence of proteasomal inhibitors (Figure 4F). However, the fusion protein was inactive in suppressing the petal overgrowth of *bb-1* (Figure 4G).

We tested for E3 activity of recombinant BB in in vitro ubiquitin-ligase assays, using *Arabidopsis* UBC10 as E2 because it interacted with the BB RING finger in a yeast two-hybrid screen and is expressed throughout the plant similar to *BB* itself (Figures S7A and 7B). Depending on the presence of both E1 and E2, His_6-tagged BB protein catalyzes the formation of high-molecular-weight ubiquitin chains (Ub_n; Figure 4K, lanes 1, 2, and 5). This activity is abolished by the C197S,C200S mutations in the BB RING-finger domain (lane 6). Using an antibody against the tagged BB protein reveals that at least some of the ubiquitin chains assemble on BB itself (Figure 4L). Both E3 activity and apparent autoubiquitination were also observed when a GST-BB fusion protein was used (data not shown). BB autoubiquitination is consistent with the rapid proteasomal turnover of the BB-GUS fusion protein in transgenic plants. Thus, BB is a functional E3 ubiquitin ligase, and mutations that abolish this activity also eliminate biological activity in planta, suggesting that BB acts by targeting cellular proteins for proteasomal degradation.

In summary, our data suggest a model in which the duration of plant organ growth and ultimately organ size is limited by the active proteasome-mediated elimination of factors that stimulate cellular growth and consequently cell division. On the basis of its expression pattern and activity as an E3 ubiquitin ligase, *BB* appears to target these stimulators for degradation and thus gradually reduce their levels during the phase of active growth. As a consequence, the balance between factors that promote growth and those that limit it tips in favor of the repressors, ensuring that growth-stimulatory activity falls below a critical threshold in a timely manner and hence growth ceases (Figure 4J). Thus, the control of organ size in plants would involve the restriction of the growth phase by targeted degradation of growth-promoting factors. Future experiments to identify the substrates for BB's E3 activity promise to uncover crucial stimulators of plant organ growth and biomass accumulation and to yield important further insights into the plant organ-size checkpoint.

Conclusions

We have identified the novel E3 ubiquitin ligase BIG BROTHER (BB) as a central negative regulator of *Arabidopsis* floral organ size. *BB* activity limits organ size in a dosage-dependent manner by restricting the period of proliferative growth. Our results demonstrate the existence of an active mechanism to limit organ size, most likely via targeted degradation of growth stimulators.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, and two tables and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/3/272/DC1/.

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